

# The evolutionary importance of cell ratio between notochordal and nucleus pulposus cells: an experimental 3-D co-culture study

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Received: 7 September 2011 / Accepted: 8 September 2011 / Published online: 28 September 2011  
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## Abstract

**Introduction** Notochordal cells and nucleus pulposus cells are co-existing in the intervertebral disc at various ratios among different mammals. This fact rises the question about the interactions and the evolutionary relevance of this phenomenon. It has been described that these relatively large notochordal cells are mainly dominant in early lifetime of all vertebrates and then differences occur with ageing. Human, cattle, sheep, and goat lose the cells with age, whereas rodents and lagomorphs maintain these throughout their lifetime.

**Materials and methods** Here, we addressed the importance of cell ratio using alginate bead 3-D co-culture of bovine nucleus pulposus cells (bNPC) and porcine notochordal cells (pNCs) for 14 days using culture inserts.

**Result** We found a significant stimulation of bNPC in the presence of pNC in terms of cell activity and glycosaminoglycan production, but not for proliferation (DNA content). Relative gene expression was significantly stimulated for collagen type 2 and aggrecan.

**Conclusion** The stimulating effect of NC was confirmed and the ideal ratio of NPC: NC was found to be ~50:50. This has direct implications for tissue-engineering approaches, which aim to repopulate discs with NP-like precursor cells.

**Keywords** Co-culture · Notochord · Nucleus pulposus · Proteoglycan/DNA content · Relative gene expression

## Introduction

Notochordal cells (NC) are remnant cells originating from the notochord present in all chordates in early embryogenesis and these cells are located in the center of the intervertebral disc [6, 17, 20, 37]. With ageing, these presumably progenitor-like cells disappear in some species and in other species they persist up to adulthood [5, 28]. In human, they disappear early in childhood [19]. Strikingly, these cells co-exist with nucleus pulposus cells (NPCs) at different ratios among different vertebrate species [28]. Rodents (rats and mice) and lagomorphs (e.g., rabbits) maintain a high number of NC cells throughout their lifetime, whereas in other animals such as bovine, goat, and sheep these cells disappear early in lifetime [19, 20].

Previous study on co-culture of non-chondrodystrophoid dog cells (e.g., Greyhound) with bovine NPCs seems to point toward regulatory mechanism and positive cell–cell interaction [1, 3, 22]. It has been speculated that these cells have precursor character and might belong to the exact same cell lineage as the disc cells since there were not too many differences reported between these two lineages [27, 32]. Other research groups are convinced that these cells are originating from another cell layer than the mesoderm, but are rather ectodermal origin. Here, we hypothesised that whether there is a ratio of NC relative to NPC cells, which is most favourable for both cell populations in terms of cell activity and extracellular matrix (ECM) production and whether these cells can influence each other by secretion of soluble factors as previous experiments have been demonstrated with co-cultures of a single cell–cell ratio [1, 3].

We hypothesize that cells of these two phenotypes are possibly influencing each other by soluble cytokines released into the media and that there is a mutualism

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between these cells. Thus, we systematically co-cultured porcine coccygeal NCs (in fact a NCs + NPC mix) and bovine coccygeal NPCs at different ratios, i.e., 0, 25, 50, 75 and 100%, respectively.

## Materials and methods

### Cell source and expansion

Porcine notochordal cells (pNCs) were isolated from the nucleus pulposus (NP) tissue of 4 to 5-month-old porcine tails obtained from the local abattoir. The high percentage of NCs in porcine NP tissue was confirmed by size and the haemocytometer using bright-field microscopy (~80%). Bovine nucleus pulposus cells (bNPCs) were harvested from the NP tissue of ~1-year-old bovine tails obtained from the local abattoir. Both cells were separated from native ECM by 0.19% pronase digestion (Roche, Basel, Switzerland) for 1 h and subsequent collagenase type 2 (Worthington, London, UK) digestion overnight (~14 h) and primary culture. The NCs from porcine NP tissue were expanded in monolayer up to Passage 2, which has been previously described as non-problematic concerning de-differentiation [3]. This expansion step of NCs was necessary since the cell yield of cell isolation was much lower (~ $1 \times 10^6$  cells) for porcine coccygeal disc cells relative to the bovine tails, and ~ $8 \times 10^6$  cells per cell type were used for each co-culture experiment.

### 3-D cell encapsulation and co-culture

The cells were encapsulated at a density of  $4 \times 10^6$  cells/mL into 1.2% alginate by the application of a syringe/22G needle and by formation of ~30 µl droplets into a 102 mM CaCl<sub>2</sub> salt solution [25]. Assuming porcine NP tissue to be 100% notochordal, the cells were kept in co-culture of pNC:bNPC ratios of 0, 25, 50, 75 and 100% in serum-free defined medium, containing 100 µg/mL penicillin/streptomycin, 50 µg/mL ascorbic acid, ITS + (Sigma, Buchs, Switzerland) and non-essential amino acids (Gibco + Sigma, Switzerland). All bead-bead co-cultures were conducted in duplicate in 12-well plates, using 0.4-µm pore size, high pore density, polyethylene terephthalate (PET) track-etched culture inserts (Becton, Dickinson and Company, Allschwil, Switzerland). The co-cultures were tracked on day 0, 7, and day 14. There were four co-culture pairings (thus, each  $N = 4$  for the porcine and bovine animals).

### Metabolic activity

Cell activity of the cells in alginate beads was measured using Alamar Blue® assay (Invitrogen, Bale, Switzerland)

[2]. Two beads per condition were incubated in 500 µL of DMEM with 10% of FCS and Alamar Blue for 3.5 h in a 48-well plate. Relative fluorescence unit (RFU) was measured at an excitation wavelength of 547 nm and an emission wavelength of 582 nm using a microplate reader (Infinite 200, Tecan, Männedorf, Switzerland). RFU measured for each tissue was normalized with the amount of DNA.

### Quantification of GAG and DNA content

Alginate beads from the Alamar Blue assay were digested with papain (Sigma–Aldrich, Bale, Switzerland) overnight at 60°C. The papain-digested samples were used for glycosaminoglycan (GAG) and DNA measurement. The GAG content was measured by the modified dimethylmethylene blue (DMMB) assay (pH 1.5) [7, 11]. The absorbance of the samples added to the DMMB buffer was read at 595 nm with a spectrophotometer. GAG concentrations were calculated from a standard curve obtained with chondroitin sulfate (Sigma–Aldrich). The amount of DNA in the sample was measured with bis-benzimidol fluorescent dye (Hoechst 33258, Sigma–Aldrich). Fluorescence was detected with Hoefer DyNAQuant (Amersham Bioscience, San Francisco, CA, US). A standard curve was generated with known concentrations of calf thymus DNA (Sigma–Aldrich, Buchs, Switzerland) and the amount of DNA of each sample was calculated from the standard curve.

### Relative RT-PCR

Relative gene expression at major anabolic genes was monitored, i.e., ACAN, collagen type 1 and 2 (Col 1 and Col 2, respectively), and ribosomal 18S as a reference gene [23, 26, 34]. Due to DNA substitutions between the porcine and bovine codon sequences of one of the two primer regions, species-specific RT-PCR was designed (Table 1) for pNCs and bNPCs. Relative gene expression was estimated by the application of a threshold cycle (Ct) and calculation of  $\Delta\Delta C_t$  and the statistics of the  $2^{-\Delta\Delta C_t}$  according to Livak and Schmittgen [24].

### Statistical analyses

All data are given as relative to the pure cell population of the same culture day. Statistical significance was tested using one-way ANOVA and Bonferroni's multiple comparison test using GraphPad Prism version 5.0d, GraphPad Software, San Diego, CA, USA, <http://www.graphpad.com>. Post-hoc power analysis was run with G\*Power software [12] to determine the type II error of the statistical tests. The power analysis revealed that it was >0.80 for the gene

**Table 1** Primer sequences used for relative real-time RT-PCR

Gene	Forward (5'–3')	Reverse (5'–3')
<i>Bovine (Bos taurus)</i>		
Bt_r18S	ACG GAC AGG ATT GAC AGA TTG	CCA GAG TCT CGT TCG TTA TCG
Bt_ACAN	GGC ATC GTG TTC CAT TAC AG	ACT CGT CCT TGT CTC CAT AG
Bt_col1 A2	GCC TCG CTC ACC AAC TTC	AGT AAC CAC TGC TCC ATT CTG
Bt_col2 A2*	CGG GTG AAC GTG GAG AGA CA	GTC CAG GGT TGC CAT TGG AG
<i>Porcine (Sus scrofa domestica)</i>		
Ssd_r18S	TAG AAG GAA GAG GAA CCA T	TAA TGT CCA ACT CAC TGA AG
Ssd_ACAN	CAG TAA CTT CGT GCC TAG	GGT CCT CTA TCT CCA GTT
Ssd_col1 A2	TAT CGG AAT TAA CCA GAC A	ACA GGA TTG ACA GAT TGA

\* Denotes primer match for both species

expression tests and the Alamar Blue data using ANOVA, and it was ~70% for the DNA and GAG statistics.

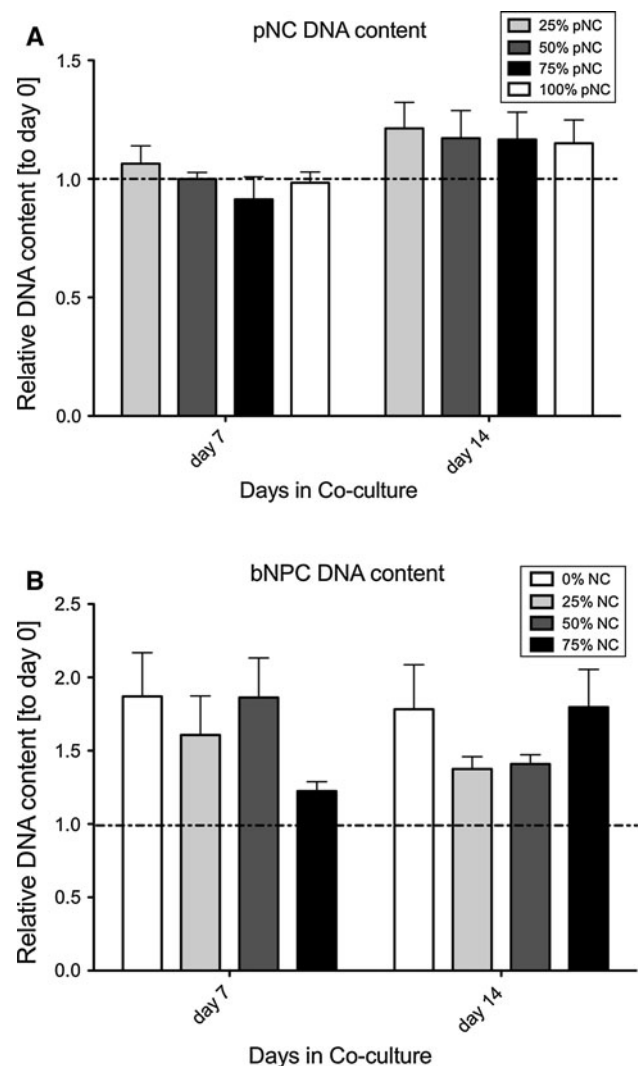
## Results

### Cell proliferation, metabolism and GAG synthesis

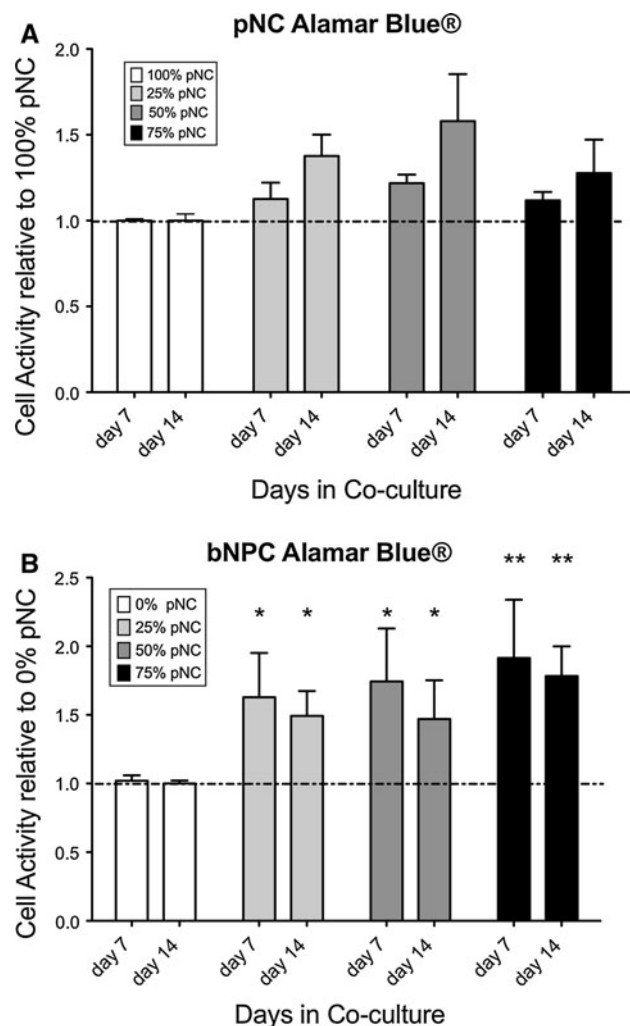
In both pNCs and bNPCs, the DNA content of beads was stable over culture time or even increased slightly relative to day 0 control after 14 days of co-culture (Fig. 1). There was no difference obvious among co-culture groups on the side of NC. There was a trend toward higher DNA content in the pure bNPC and the 25% bNPC and 75% pNC group; however, this was non-significant. As for the cell metabolism (Fig. 2a, b) the bNPCs were more strongly activated by the presence of pNCs (Fig. 2b) ( $p = 0.008$ ) than vice versa (Fig. 2a), and this effect was the strongest in 75% of pNC co-culture and was also significant using Bonferroni testing for the 0 versus the 50% group and for the 0 versus the 75% group (Fig. 1a). We found a significant increase in GAG/DNA ratio for the 50% bNPC group after 14 days of co-culture (Fig. 3b), but no effect for pNC co-cultures (Fig. 3a).

### Relative gene expression

For bNPCs, relative gene expression revealed up-regulation of ACAN by two to five times and slight up-regulation of Col 2 (Fig. 4b). It was most strongly up-regulated in the 50% pNC co-culture group. For pNCs, ACAN and collagen type 2 were found to be up-regulated by about 1,000 times, however, this effect was not significant for both mRNAs (Fig. 4a). This up-regulation by a factor of ~1,000 times for all three co-culture ratios containing bNPCs could be interpreted as a progenitor-like cell status of pNCs prior co-culture. On the other hand, Col 1 was found unchanged for the entire duration of the co-culture experiment, indicating maintenance of the original phenotype during the experiment.



**Fig. 1** DNA content of the alginate bead after 7 and 14 days co-culture for **a** porcine notochordal cells (pNC) and **b** for bovine nucleus pulposus (bNPC).  $N = 4$  co-cultures repeats, plot of mean  $\pm$  SEM



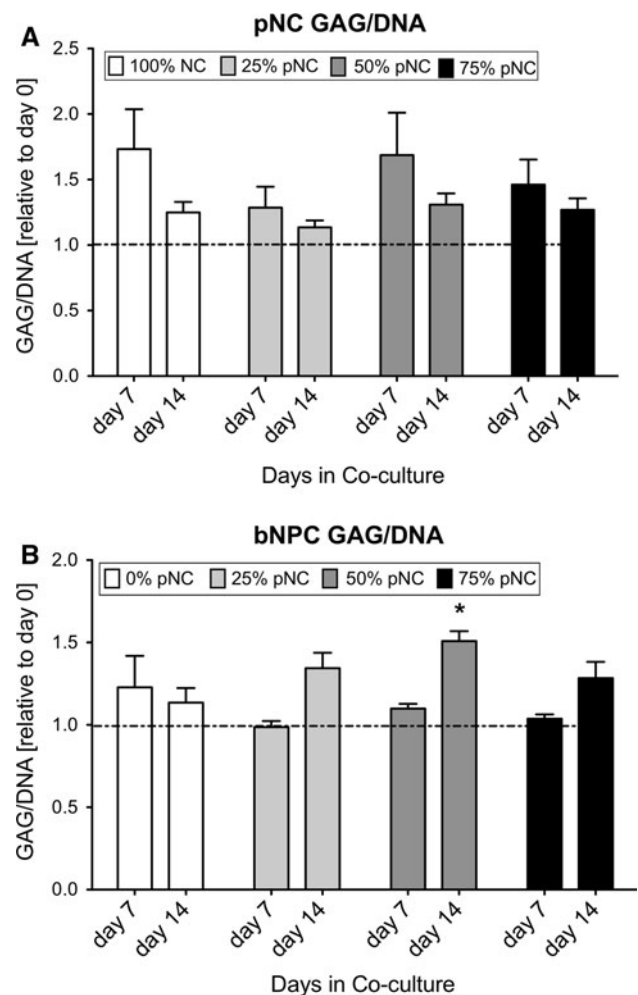
**Fig. 2 a, b** Cell metabolism per DNA (cell) in porcine notochordal cells (pNCs) and bovine nucleus pulposus cells (bNPCs) cultured in 3-D alginate for 7 and 14 days, respectively. Plot of mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$

## Discussion

### Cell identity of notochordal cells

The notochordal cells (NC) are entrapped during early embryogenesis (around day 20 in humans) and formation of the somites. This formation of the so-called prototissue which forms at the center of the embryo appears as a rod of tissue known as the notochord, which guides the embryonic development of the neural tube and the vertebral column, including the intervertebral disks [31]. The exact role of the entrapped notochordal cells after condensation is unclear, but they are believed to take part in the formation of the nucleus pulposus [4, 17, 37].

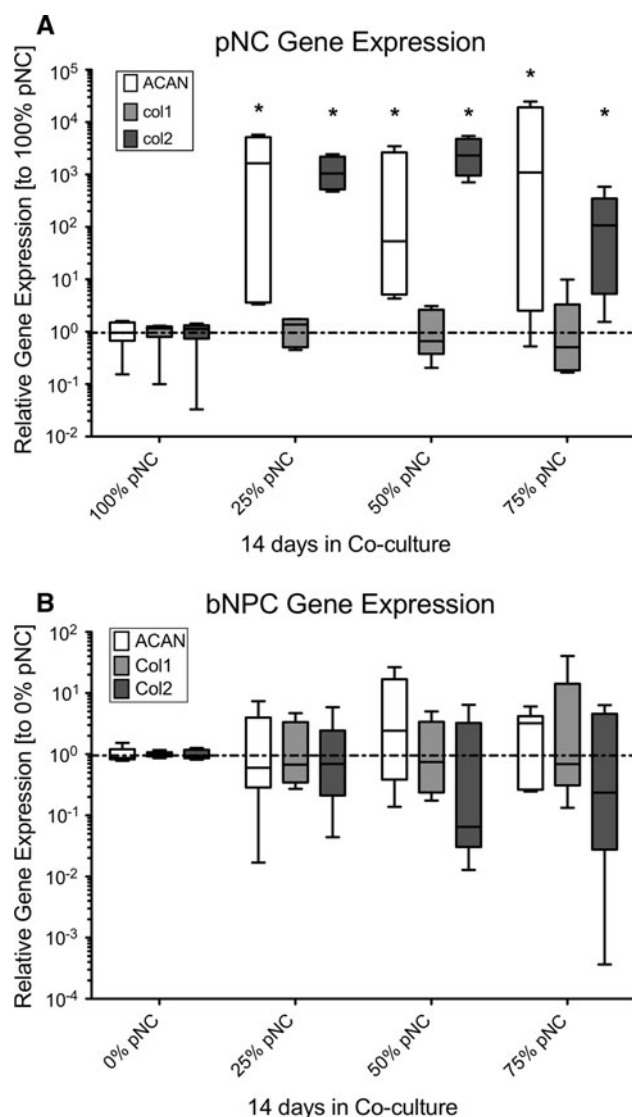
However, it seems obvious that these two cell populations differ in a number of characteristics such as cell size, nutrition [16], surface markers [13], and mechano-sensitivity



**Fig. 3** GAG/DNA ratio of **a** porcine notochordal cells (pNCs) and **b** bovine nucleus pulposus cells (bNPCs).  $N = 4$  co-cultures repeats, mean  $\pm$  SEM. \* $p < 0.05$

[14]. Recently, the physiological requirements in terms of nutrition were compared between these two cell types and it was found that notochordal cells are more sensitive and consume more glucose than nucleus pulposus cells under the identical culture condition [16]. Furthermore, non-invasive femtosecond laser microscopy revealed clear size differences between these two cell types and seems to point toward two different cell lineages, if cell shape and size are considered [15]. Furthermore, the NC differs by the presence of large vacuoles, which can be separated by the size-scatter of FACS analysis [5]. These large vacuoles found in NC of the intervertebral disc has been attributed a possible functional role in osmoregulation [18].

There are also considerable differences in the nucleus pulposus cell shape among different animal species [19, 20]. Of interest are especially the two dog breeding lines, i.e., the chondrodystrophoid dogs, e.g., Dachs hound and Beagle and the non-chondrodystrophoid dogs, e.g., Mongrels, Greyhound, and German shepherd lines. There have been several



**Fig. 4** Relative Gene Expression after 14 days in co-culture of bNPCs and pNPCs in 3-D microspheres in alginate, respectively.  $N = 4$  co-cultures repeats, mean  $\pm$  SEM. \* $p < 0.05$

morphological papers published, which describe the morphological differences between these cells [19, 20].

Transcriptomics, on the other hand, comparing the two cell populations revealed that there are only about two dozen genes really distinct between these two cell types [27, 30]. The search for specific markers to distinguish these two cell populations has just started [13]. Weiler et al. [38] found that cells in the human fetal and juvenile nucleus pulposus with the typical morphology of the notochord (physaliferous) express the markers cytokeratin (CK)-8, -18, -19 and galectin-3 [29]. Gilson et al. [13] found that pig NP cells, which are phenotypically similar to human infant nucleus pulposus cells, were all CK-8 positive. In human discs, the presence of notochordal cells has been associated with the occurrence of chordomas, which are malignant tumours that

occur along the spine [36]. Brachyury (i.e., T gene) is a transcription factor associated with the notochord. It has been demonstrated that brachyury positive (T+) cells are associated with chordoma. Interestingly, human chordoma express high levels of aggrecan, collagen type 2, but lack expression of collagen X completely [36]. T+ cells are also related to cancer stem cells and express nanog and other stem cell-related markers and have been described as cells with higher “plasticity” [33].

It has also been shown that notochordal cells are perfectly adapted to low-oxygen environment. They can produce a better aligned ECM under hypoxic conditions [10]. Here, we cultured the cells under normoxic conditions. It is very likely that the current results would be even more pronounced under hypoxic conditions [10]. This should be tested in a further experiment.

#### Optimal cell ratio

We could demonstrate a significant increase of cell activity of the bNPCs in the presence of pNPCs and activation of GAG/DNA ratio by a ratio of 1:1. The strongest activation of cell activity was found with a ratio of  $\sim 75\%$  pNPCs. Considering that the porcine nucleus pulposus contains not 100% notochordal cells, but to some extent, i.e.,  $\sim 10\text{--}20\%$  of pNPCs, the real ratio might have been shifted toward NPCs and thus was around 30:70 for pNPC:bNPC. It is of interest that NPCs reacted positively to the addition of bovine NPCs since the “pure” population was so to say a natural “co-culture” of conspecific NCs and NPCs. The fact that cross-specific cell co-culture results in an activation of the notochordal cells could be either an artifact of the cross-species cytokines or a true effect pointing to a precursor state of notochordal cells, which can be triggered toward higher expression of aggrecan and collagen type 2 by the presence of additional NP cells. The fact that the DNA content in pNPC was not significantly higher than in the bNPCs suggests that the expansion phase with serum in monolayer of the NCs did not bias the results of the GAG/DNA ratio. The fact that a 50% ratio of pNPCs and bNPCs increased significantly GAG/DNA ratio of bNPCs exclusively is interesting with respect to the existence of pre-established regulator mechanisms, which can be triggered by reaching an optimum interaction between the two cell populations. Addition of bNPCs to the NPC population resulted in relative increase of cell metabolism in bNPCs (Fig. 2b). This activation is in line with the increase of GAG/DNA over time on the side of NPCs. GAG/DNA ratio on the side of NCs was elevated (around 50% higher than day 0) after 7 days and then were reduced to the level of the start of the co-culture. This is a contradiction to the gene expression results, which show an increase of aggrecan and collagen type 2.



Future experiments will test the influence of cell–cell contact by seeding pNCs and bNPCs into alginate beads enabling cell–cell interaction and also the importance of hypoxic conditions. Gene expression revealed a significant up-regulation of Col 1 and Col 2 by the pNCs in the presence of bNPCs, which confirms previous findings of Boyd [3] and Aguiar et al. [1] that NCs express certain stimulating cytokines, which seems evident and has been demonstrated using conditioning media [3, 9, 22]. Korecki et al. also detected that SOX-9 (transcription factor for chondrogenesis) was down-regulated if human mesenchymal stem cells (hMSCs) are exposed to notochordal conditioned medium (NCM) as compared to a chondrogenic medium containing TGF- $\beta$ . NCM, however, also increased expression of collagen type 2, but not to the same extent as in the presence of TGF- $\beta$ . They also concluded that NCM tended to increase laminin  $\beta$ 1 mRNA expression, and also found significantly higher level of GAG production than for TGF- $\beta$ -treated cells. In addition, NCM conditioning of MSCs tended to up-regulate collagen type II mRNA less strongly than with TGF- $\beta$  from which the others concluded that NCM has the potential to thrive cells toward the “discogenic” phenotype rather than TGF- $\beta$  alone. Interestingly, NCM stimulated the migration of IVD cartilage endplate chondrocytes in in vitro cell migration assays [21].

## Conclusion

In conclusion, co-culture of porcine notochordal cells (pNC) together with bovine nucleus pulposus cells (bNPC) definitively stimulates both cells in a synergistic way. However, our data also show that bNPC are activated by the presence of pNC, which results in a higher GAG per cell production (significant in the case of a cell ratio of 1:1) and higher cell activity as measured by Alamar Blue assay. The NPC, on the other hand, neither activate the NC proliferation nor the GAG/DNA ratio, but instead the gene expression of collagen type 2 and aggrecan. We interpret this result as an indication that the notochordal cell phenotypes might have been at a progenitor state before the co-culture experiment. These data do not contradict the latest hypotheses about their origin being very related cell population to the chondrocyte-like disc cells [30]. The fact that the co-culture with cross-specific additional NPCs cells can trigger aggrecan and col 2 gene expression seems to point toward a progenitor-like status of notochordal cells. Moreover, these cells might be of key importance for the regeneration of the intervertebral disc using cell-based approaches [8, 13]. Although these notochord cells seem to be highly related to chondrocyte-like cells, the differential response in our co-culture experiments questions whether

notochordal cells are irrelevant for the choice of animal models for disc regeneration [30, 35]. Although NC have stimulating effects on other IVD cells their implication for therapeutic usage for the human IVD can only be followed-up after clarification of their phenotypic status and potential to form chordoma. Much easier might be the clinical application of their yet unknown cytokines/substances which they secrete for potential medication.

**Acknowledgments** This work was supported by the Swiss National Science Foundation (SNF #310030-127586/1) and the Department for Orthopaedic Surgery, Insel University Hospital, Bern.

**Conflict of interest** None.

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